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OSTEOARTHRITIS and CARTILAGE

Ultrastructural immunolocalization of bone sialoprotein in guinea-pig osteoarthritis

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Summary

In diarthrodial joints, bone and cartilage are structurally and functionally inseparable as shown in osteoarthritis (OA), where subchondral bone changes are integral in the disease process. By ultrastructural immunohistochemistry using polyclonal antibodies against guinea-pig bone sialoprotein (BSP), we investigated the distribution of this matrix protein at the osteocartilaginous interface in Hartley guinea-pig knees at different stages of primary osteoarthritis. Between 6 and 12 months they developed moderate osteoarthritic changes predominantly in the medial condyle, progressing to severe OA at 30 months. In all age groups BSP labeling was concentrated to the osteocartilaginous interface at a 1 μm narrow zone at the interface. In the medial osteoarthritic condyle, BSP was increased as compared with the lateral nonosteoarthritic condyle, but only at 30 months, when cartilage fibrillation correlated to BSP. Our observations suggest that altered BSP abundance may be a potential bone marker for late stage OA, while early events in bone cannot be monitored. BSP is expressed early in osteogenesis and may have a role in biological mineralization and growth. Since a sharp zone of intense BSP labeling remains at a remarkably constant level throughout life in guinea-pigs, BSP may have an important structural and/or regulating role at the interface. The protein may act as an anchor of calcified articular cartilage to subchondral bone or by regulating mineralization at the osteocartilaginous interface.

Key words: Bone sialoprotein, Guinea-pig, Immunohistochemistry, Osteoarthritis.

Introduction

Clinically, osteoarthritis (OA) has been described as an unspecific end stage of organ failure [27]. Various animal models of OA have been designed [1]. Most of these models involve an intra-articular intervention resulting in rapidly progressive changes, quite unlike primary OA. There are, however, naturally occurring OA-like conditions in animals, e.g., Hartley guinea-pigs [4]. The importance of subchondral bone changes in OA pathogenesis has previously been stressed [10, 27]. Early subchondral bone trabecular thickening simultaneous with cartilage changes have been shown by microscopic computed tomography [11]. Clinically, subchondral sclerosis is the first reliable radiographic sign of OA and it has been suggested to preceed joint space narrowing [25]. Magnetic resonance imaging has shown a consistent increased thickness of sclerotic bone with osteoarthritis [35]. The need for molecular markers for monitoring the OA process is evident [13]. Work has been concentrated on cartilage markers [6, 18], whereas markers of bone turnover have until recently mainly been used to monitor other diseases like osteoporosis [12].

Bone contains several proteins with high contents of sialic acid-containing oligosaccharides which may regulate matrix assembly and mineralization [16]. These include, bone sialoprotein (BSP) and osteopontin, which have both been purified and characterized during the last decade [15].

BSP is rather restricted in its distribution to bone, with undetectable levels of expression in most other tissues, with the possible exception of hypertrophic chondrocytes [7, 9]. Its an acidic, phosphorylated and sulfated glycoprotein, rich in sialic acid. The core protein has a molecular weight of approximately 32 kDa. In-situ hybridization studies have demonstrated BSP mRNA early in bone formation and BSP has therefore been suggested to have a role in early osteogenesis [19]. The protein has also been ascribed a role in

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biological mineralization [7]. Clinically, BSP shows increased turnover in joint disease, and increased amounts are released into the synovial fluid in rheumatoid arthritis [29], and into serum in OA [26].

Since tissue compartments containing BSP appear to show altered turnover in joint disease, we have utilized studies of BSP release as an indicator of early involvement of the osteocartilaginous interface in guinea-pig OA.

Materials and Methods

BSP ISOLATION

Diaphyseal bones from the lower limbs from 2 month-old Dunkin Hartley guinea-pigs ($N = 10$), killed by intraperitoneal injections of pentobarbital, as approved by the Local Animal Care Ethical Committee, were frozen, powderized and kept in −20°C over night. The bone powder (50 g) was extracted sequentially with 10 volumes of 4 M guanidine/HCl, 50 mM sodium acetate, 0.1 M ε-ACA, 5 mM benzamidine, pH 5.8, followed by extraction in 30 volumes of 4 M guanidine/HCl, 50 mM Tris/HCl, 0.1 M ε-ACA, also containing 0.5 M-trisodium EDTA, and 5 mM benzamidine, pH 7.4 [14]. Both extraction solvents were supplemented with proteinase inhibitors and with 5.4 mM N-ethylmaleimide [17]. The second extract was then concentrated at 4°C by ultrafiltration over a PM-10 filter (Amicon Corp., Lexington, MA, U.S.A.). The material retained was brought into 7 M urea, 0.1 M sodium acetate, 10 mM Tris/HCl buffer, pH 6.0, by diaflow with 10 volumes of the urea solution.

The guanidine HCl/EDTA-extract, brought into 7 M urea/Tris buffer was chromatographed on a DEAE-sepharose ion-exchange column (Whatman Chemicals, Maidstone, Kent, U.K.) [15]. Bound material was eluted with a linear salt gradient (500 ml) ranging from 0–1.0 M sodium chloride in 7 M Urea, 10 mM Tris, pH 6.0. Fractions of 15 ml were collected and analyzed for protein by their absorbance at 280 nm.

Representative samples from the fractions were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions to identify the elution position of BSP. Gradient polyacrylamide (4–16%) slab gels which were cast with a 4% stacking gel and SDS buffer system were used [22]. Fractions containing predominantly BSP, were pooled and taken to immunization of rabbits to prepare polyclonal antibodies.

ANTIBODY PREPARATION

Antiserum was raised against BSP by immunizing rabbits with the isolated fractions containing BSP (50 μg), as approved by the Local Animal Care Ethical Committee. Initial immunization was with Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.) and subsequent boosters, after 4 and 8 weeks, were with Freund's incomplete adjuvant (Difco Laboratories).

SDS-PAGE and Western blot analyses were used to test the specificity of the antibodies. A sample of the crude guanidine HCl/EDTA extract was precipitated with ethanol and electrophoresed on a gradient SDS-polyacrylamide gel, as described elsewhere [29]. Proteins in the gel were electrophoretically transblotted to nitrocellulose and processed for positive immunodetection with the antiserum, essentially as described by Towbin [33] using a 1:200 dilution of rabbit anti-guinea-pig BSP and a 1:500 dilution of peroxidase conjugated pig anti-rabbit IgG (DAKO, Denmark). Only one immunoreactive band was observed with the guanidine HCl/EDTA extract of guinea-pig bone. Its migration corresponds to that of BSP.

ULTRASTRUCTURAL IMMUNOHISTOCHEMISTRY

Three outbred male Hartley guinea-pigs (Møllegård, Copenhagen, Denmark) from each of three age groups: 6 (adult), 12 (middle aged) and 30 (old) months of age were used. Mean (SD) body weights were 960 (60) g, 1160 (50) g, and 1230 (90) g. The animals were heparinized (Lövens läkemedel, Malmö, Sweden), anesthetized by fentanyl–fluanison/H_2O (Hypnorm, Leo, Helsingborg, Sweden) and fixed by vascular perfusion as above using 0.1 M phosphate-buffered fixative of 0.3% glutaraldehyde and 0.3% para-formaldehyde, pH 7.4, containing 3% dextran T40 (Pharmacia, Uppsala, Sweden). Subsequently the proximal tibiae were dissected out. The specimens were cut sagittally into two approximately equal portions, i.e., medial and lateral plateaus. The central compartment comprised about 30% of the joint surface. Thin vertical slices including the cartilage and a minimal part of the subchondral bone were cut into small pieces and further fixed by immersion in the same fixative for 2 h at room temperature. The specimens were dehydrated in methanol and embedded at low temperature in the polar resin Lowicryl K11M (Chemische Werke Lowi GmbH, Waldkreiburg, Germany) [20]. Two ultrathin sections (35–40 nm) were cut from two independent blocks from each animal. The specimens were placed on formvar-coated nickel grids. After

blocking with 1% bovine serum albumin (BSA) and 0.1% gelatin, the sections were incubated with antibodies in phosphate-buffered saline containing BSA [20]. All sections used for immunohistochemistry were incubated simultaneously using the same stock of solutions. Positive immunostaining was detected by protein-A coated with 10 nm gold (Amersham, Buckinghamshire, UK). Subsequently, the sections were contrasted with 4% uranyl acetate and lead citrate.

MEASUREMENTS

Electron micrographs were sampled along the cartilage and the osteoid interface in articular cartilage by systemic random sampling [9]. The distributions of immunogold markers for BSP were studied on printed copies at a final magnification of 65 000. Three compartments were defined in the proximal epiphysis at the lower border of articular cartilage: mineralized cartilage, interface and bone. The interface was defined as the area between the cartilage–bone interface and a line drawn at a distance of 1 μm deep into the bone matrix (Fig. 2). Cartilage and bone compartments were defined as compartments on each side of the interface at an approximate distance of 5 μm and parallel to the border region [9].

The number of animals (3), blocks (3) and sections (1) were chosen after a pilot study using cumulative mean plots for evaluation. Eight micrographs were taken from each of three compartments from each section. In all, the data were based on more than 1300 micrographs. The labeling per unit area in each compartment in the medial and lateral condyles was calculated on printed copies. In addition, for ultrastructural analyses of the BSP labeling within the border region, the distance of each gold particle from the osteocartilaginous interface was measured with a semiautomatic image analyzer (Videoplan, Zeiss, Germany). The 1 μm thin osteocartilaginous interface was divided into five equal intervals, each 0.2 μm thin.

Before embedding, sections for light microscopic evaluation were also made. Three to four decalcified sections from each condyle from each animal were cut and stained with toluidine blue. Articular cartilage fibrillation and the calcified cartilage thickness were chosen as parameters of OA. Both have previously been reported to be increased in OA [10]. Cartilage fibrillation was measured by intersection counting with a cycloid grid for vertical sections [3], i.e., the number of intersections through the item in question, divided by the number of intersections with the smooth contour of the joint surface. The thickness of the calcified cartilage was measured perpendicular to the joint surface.

Analysis of variance, Tukey's test for correction for multiple comparisons, Kolmogorov–Smirnov nonparametric test, and Pearsons test for correlation at a rejection level of 5% were used for statistical analyses.

Results

The 6-month-old animals showed no gross alterations typical for OA, i.e. surface fibrillation, cartilage destruction or bone sclerosis. However, at 12 months the central nonmeniscus covered portion of the medial plateaus had developed typical OA alterations, with a roughened cartilage surface, subchondral bone sclerosis and osteophytes at the joint margins (Fig. 1). In contrast, the lateral surface was smooth without signs of OA. In the medial condyle, the changes progressed, and at 30 months there was subchondral bone eburnation. In the lateral condyle, only mild fibrillation was observed at 30 months.

Calcified cartilage was always thicker in the lateral condyle compared with the medial (Table I). With increasing OA, calcified cartilage thickness increased both in the medial and lateral condyles, but without statistical significance in the lateral (non-OA) condyle. Cartilage fibrillation increased with progressive OA in the medial condyle, while in the lateral condyle values were low and rather

FIG. 1. Histological section from the central nonmeniscus covered portion of the medial plateau showing osteoarthritic (OA) changes in the guinea-pig proximal tibia, with cartilage fibrillation (F), increased calcified cartilage thickness (C), and subchondral bone sclerosis (S) at 12 months of age. The lateral plateau was not affected by OA. At 30 months of age, the changes progressed to severe OA and included osteophytes at the joint margins. (Original magnification ×125).

Table I
Thickness of calcified cartilage in the central medial osteoarthritic (OA) and lateral (non-OA) condyles in different ages in guinea-pig proximal tibias

Age	Medial	Lateral
6 months	0.08 (0.01)*	0.15 (0.01)
12 months	0.13 (0.02)†	0.18 (0.03)
30 months	0.12 (0.01)‡	0.18 (0.02)

Mean values (S.D.) mm ($N = 3$). *$P < 0.05$ between medial and lateral condyle 6-months-old groups. †$P < 0.05$ between 6- and 12-months-old groups. ‡$P < 0.05$ between 6- and 30-months-old groups.

constant (Table II). BSP immunolabeling showed low levels in cartilage, a very sharp, several-fold increase at the interface, and then a sharp decrease in bone to levels three times those in cartilage (Fig. 2). In the lateral (non-OA) condyle, BSP immunolabeling decreased in the oldest group in the interface (Table III). There were no changes observed medially (Table III). The ultrastructural BSP distribution is displayed in Fig. 3. There was a correlation between cartilage fibrillation and BSP immunolabeling in the osteocartilaginous interface in the medial condyle (severe OA) in the 30 months group ($r^2 = 0.94$). However, we could not observe any correlation between calcified cartilage thickness and BSP labeling, nor between fibrillation and BSP immunolabeling in articular cartilage and subchondral bone.

Discussion

The restricted BSP distribution to the osteocartilaginous interface corroborates previous findings from normal rat tibias [9]. In young rats with rapid bone turnover, BSP labeling increased about twofold between 21 and 32 days of age and then remained at the same level at 84 days [9]. In this study we found that the sharp gradient of BSP between cartilage and bone remained largely unchanged throughout life, with only a slight decrease with age in the lateral non-OA condyle in the oldest animals. This may be explained by the

Table II
Articular cartilage fibrillation in the central medial (OA) and lateral (non-OA) condyles

Age	Medial	Lateral
6 months	1.1 (0.1)	1.0 (0.0)
12 months	2.0 (0.2)*	1.2 (0.0)
30 months	1.7 (0.2)†	1.2 (0.2)

Mean values (S.D.) mm/mm ($N = 3$). *$P < 0.05$ between 6- and 12-months-old groups. †$P < 0.05$ between 6- and 30-month-old groups.

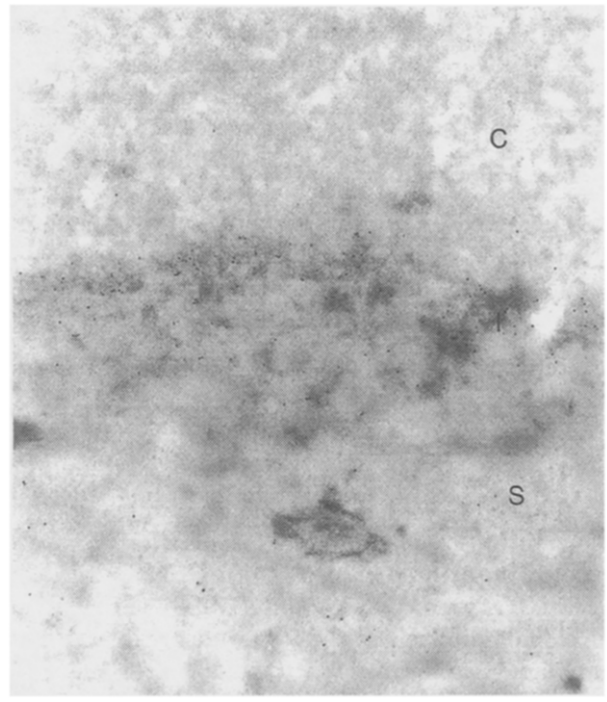

FIG. 2. Electron micrograph of bone sialoprotein (BSP) immunolabeling from a 12 month-old guinea-pig (proximal tibia). BSP immunolabeling was highest in the interface (I) between cartilage and bone, lower in subchondral bone (S), and at background levels in cartilage (C). (Original magnification ×65 000).

fact that old guinea-pigs have less bone turnover, as compared with the relatively younger rats, which still exhibit bone growth and higher bone turnover. As discussed elsewhere [19], it is likely that BSP binds to components at the interface, becoming fixed at this site. The roles this confer to BSP are unclear, but the protein may regulate processes at the interface, and/or act as an anchor of calcified articular cartilage to subchondral bone, e.g., regulating mineralization at the osteocartilaginous interface.

BSP labeling was higher in the osteocartilaginous interface in the medial condyle with advanced OA as compared with the lateral condyle with only superficial fibrillation. This is in line with previous studies implicating the importance of subchondral bone changes in the OA pathogenesis [27]. However, there was no difference in BSP labeling between condyles in the 12-month-old group with moderate OA changes. These findings suggest that BSP may be a bone marker for late stage OA, while early events in bone do not result in altered deposition of BSP. This is supported by the fact that serum BSP is increased in patients with radiographic OA, but normal at an earlier stage without radiographic pathology [26]. In severe rheumatoid arthritis (RA), BSP in synovial

Table III
Distribution of BSP immunolabeling in different compartments in the central medial (OA) and lateral (non-OA) tibial condyles

	Cartilage		Interface		Bone	
Age	Medial	Lateral	Medial	Lateral	Medial	Lateral
6 months	0.4 (0.2)	0.3 (0.1)	9.5 (0.4)	9.3 (0.1)	1.0 (0.3)	1.3 (0.1)
12 months	0.4 (0.2)	0.3 (0.1)	9.3 (0.5)	9.5 (0.1)	1.2 (0.4)	1.0 (0.1)
30 months	0.4 (0.1)	0.6 (0.4)	9.4 (0.1)‡	8.9 (0.1)*†	1.2 (0.1)	1.3 (0.2)

Mean value (S.D.) for goldmarkers per area unit ($N = 3$). *$P < 0.05$ between 6 and 30 months groups. †$P < 0.05$ between 12 and 30 months groups, ‡$P < 0.05$ between lateral and medial condyles.

fluid is increased with progressive joint damage [29]. This is probably the result of a much more rapid degree of bone destruction in RA as opposed to OA. BSP concentration increases in patients with progressive OA, but remains constant in patients showing no progression [26]. This may indicate that the early changes in subchondral bone may be related to altered turnover, rather than altered composition, in view of the rather constant BSP-levels locally.

The distinct feature of bone changes in OA have been pointed out [27]. Alterations in trabecular subchondral bone have been found as early as the first histologic cartilage changes detectable by the use of microscopic computed tomography in studies of the hindlimb myectomy OA model [11]. The progression of bone pathology in OA, as measured by increase in subchondral bone thickness, has been verified by magnetic resonance imaging of developing changes in guinea-pig OA [35].

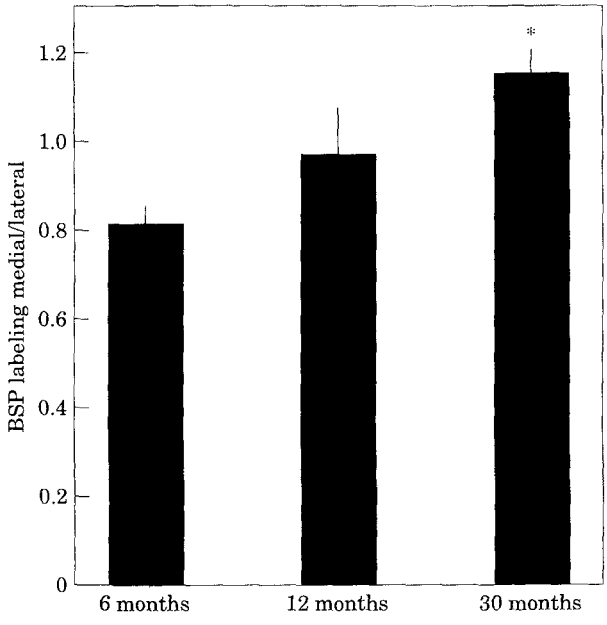

FIG. 3. Bars illustrating the BSP immunolabeling within the 0–0.2 μm interval of the interface between articular cartilage and subchondral bone. The ratio BSP labeling medial/lateral condyle increased with increasing OA between 6- and 30-month-old groups (*$P < 0.05$). Mean values (S.D.) for goldmarkers per area unit ($N = 3$).

The normal osteocartilaginous interface is irregular and coral-like. In guinea-pig OA this surface flattens [8], most likely as the result of an ongoing remodeling. If BSP labeling is related to the surface density of the interface, the difference between the medial and lateral condyles at 30 months further increases. This is consistent with the suggested early event in OA being a pathologic disturbance in tissue remodeling activity at the cartilage–bone interface [21], where there is a marked concentration of shear stress [5, 23]. Subchondral bone stiffening increases the shear stresses in the overlying articular cartilage [2].

We observed a correlation between BSP labeling and cartilage fibrillation [10], which corroborates the notion that cartilage and bone changes are parallel in OA. Coincident changes in articular cartilage and subchondral bone in progressive OA have been observed by bone scintigraphy and measurements of serum levels of cartilage oligomeric matrix protein (COMP) [31]. Previous studies have reported an increase of the calcified cartilage thickness in human OA [34], as well as in guinea-pig OA [8]. In OA a correlation has been shown between an increased calcified cartilage thickness and trabecular bone volume [24] which suggests an increased bone metabolism.

Although much attention has been given to biochemical markers of both bone formation and bone resorption in different metabolic diseases such as osteoporosis [12], studies in OA have been scarce [26]. Markers such as osteocalcin [30] and pyridinium cross-links of collagen appear to have limited applications in early OA [28]. In cartilage there is an abundance of potential specific matrix markers, COMP [31], keratan sulfate [for review see 32], and novel epitopes, i.e., chondroitin sulfate fragments such as 3B3 [6]. However, in view of the increased importance of bone changes in the OA pathogenesis, further studies of OA bone markers are clearly needed.

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